Molecular Biology Reports (2019) 46:77–82
https://doi.org/10.1007/s11033-018-4447-6

ORIGINAL ARTICLE

ARA lncRNA, is upregulated in liver and breast tumor tissues

Farzaneh Raeisi[1] · Mahdieh Abolfathi[2] · Raheleh Ahmadi-Naji[3] · Sara Iranparast[4] · Esmat Noshadi[5,6] · Arvand Akbari[5,6] · Esmaeil Mahmoudi[1] · Mansoor Khaledi[7] · Asghar Arshi[8]

Received: 4 July 2018 / Accepted: 17 October 2018 / Published online: 26 October 2018

Abstract

Important regulatory roles of long non-coding RNAs (lncRNAs) have been recently found, and reported as useful biomarkers in cancer. To identify a potential expression of the new discovered lncRNA (*ARA*), during promotes cell proliferation, apoptosis inhibit, migration and cell cycle arrest, we firstly evaluate its expression in two cancer tissues (breast cancer and liver cancer) and then compared its variability expression in tumor versus non-tumor samples. Expression profile of *ARA* lncRNA was evaluated using qRT-PCR in paired tumor and marginal non-tumor samples collected from patients who had been referred to the Shiraz General. After RNA extraction from tissue samples, cDNA synthesis and RT-qPCR method were performed according to the protocols. *ARA* lncRNA expression level was calculated using $2^{-\Delta\Delta Ct}$ method. Principal-component analysis followed by receiver operating characteristic curve analyses was performed to evaluate the diagnostic potential of selected lncRNA. Our data revealed a significant upregulation ($P < 0.001$) of *ARA* in breast and liver tumor tissues, in comparison to same patients non-tumor marginal samples. Also, there was a significant difference between the expression of *ARA* lncRNA in breast cancer and liver cancer patients ($P < 0.05$). In conclusion, the results of our study suggest a possible role of *ARA* lncRNA in proliferation of breast and liver tissues, as well as its potential usefulness as a novel diagnostic biomarker for breast and liver tumors.

Keywords LncRNA · *ARA* · Breast cancer · Liver cancer

Introduction

Cancer is a major public health problem and one of the leading causes of death worldwide. Different cancer types have been influenced by some important factors such as age, gender, race, environmental factors, diet, and genetics [1]. Today breast cancer, the most common malignity, is a significant challenge to human health which causes females death (estimated 40,160 deaths in 2017, USA) all over the world and has expanded over the past 30 years [2–4]. Breast cancer which is a multi-stage process characterized by changing genetic and epigenetic and also affect the growth and development in main cellular pathways [5]. Several epigenetic changes and genomic polymorphisms in genes controlling the circadian rhythm have been shown to be significantly associated with cancer development. The incidence of liver cancer worldwide is different. There are major risk factors for liver cancer including, infection with HBV and HCV (hepatitis B and C virus), exposed to certain chemicals, intake of alcohol and metabolic diseases e.g. obesity and diabetes [6, 7]. There are two histological

✉ Asghar Arshi
asghararshi@iaushk.ac.ir

1 Young Researchers and Elite Club, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

2 Department of Immunology, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

3 Student Research Committee, Shahrekord University of Medical Sciences, Shahrekord, Iran

4 Department of Immunology, Ahvaz University of Medical Sciences, Ahvaz, Iran

5 Department of Biology, Faculty of Science, Fars Science and Research Branch, Islamic Azad University, Marvdasht, Iran

6 Department of Biology, Faculty of Science, Marvdasht Branch, Islamic Azad University, Marvdasht, Iran

7 Medical Microbiology, Student Research Committee, Shahrekord University of Medical Sciences, Shahrekord, Iran

8 Young Researchers and Elite Club, Najafabad Branch, Islamic Azad University, Najafabad, Iran

types of liver cancer, hepatocellular carcinoma (HCC, one of the most deadly cancers worldwide, derived from hepatocytes) and cholangiocarcinoma (derived from the intrahepatic bile ducts epithelial lining) [8, 9].

Indeed, the interference between protein-coding genes and noncoding RNAs (ncRNAs) is necessary in the molecular network that regulates development and diseases [10]. NcRNAs can be classified by their size, such as short ncRNAs, midsize ncRNAs and long noncoding RNAs (lncRNAs) (19–31, ~ 20–200 and > 200 nucleotides, respectively). The functional importance of lncRNAs in mammalian development and diseases was recognized and systematic functional and genomic studies showed that lncRNAs dysregulation has been associated with cancer development [11, 12]. Jiang et al. [13], performed a microarray analysis of lncRNA in adriamycin resistant MCF-7/ADR breast cancer cells in comparison with parental MCF-7 cells and a lncRNA was validated with specific differentially expressed that called lncRNA-*ARA* (adriamycin resistance associated).

ARA lncRNA is derived from an intron of p21-activated kinase 3 (PAK3) gene at chromosome Xq23 and playing a role in promotes cell proliferation, migration, apoptosis inhibition and cell cycle arrest [13, 14].

Recently, molecular techniques, especially gene expression profiling, have been used increasingly, in order to, biomarker-based prediction and prognosis, understanding the molecular mechanisms, therapeutic strategy development and eventual improvement of patient outcomes. Therefore, the aim of this study was to evaluate *ARA* lncRNA expression by a quantitative reverse transcription PCR (qRT-PCR) in breast and liver cancer tissues, to explore its clinical significance and to assess the potential value of *ARA* lncRNA as a prognostic marker.

Materials and methods

Cancer sample collection

15 breast cancer samples and 15 liver cancer samples plus 15 normal tissues of each cancer were received from patients referring to Shiraz and Isfahan General Hospitals. Samples were obtained from patients undergoing hysterectomy without preoperative chemotherapy or radiotherapy, and were histologically evaluated for type and grade. All samples were transferred to RNAlater immediately after resection and stored at − 20 °C until used for RNA extraction. Informed consents were obtained and the experimental procedure was approved by the Human Studies Committee of Islamic Azad University, Shahrekord Branch, Shahrekord, Iran with 17621105 approval number.

RNA extraction and cDNA synthesis

Total RNA was extracted from tissue samples using the RNX^{TM}-Plus solution (SinaClon, IRAN) according to the manufacturer's instructions, except for an extended 1-h treatment with DNaseI. For checking RNA purity, concentration and integrity, Thermo Scientific NanoDrop™ 1000 Spectrophotometer and electrophoresd on 2% agarose gel were used respectively. One microgram of RNA was used for complementary DNA (cDNA) synthesis by using random hexamer priming and PrimeScript^{TM}-RT reagent kit (TaKaRa, Japan) and its concentration was then checked spectrophotometrically.

Quantitative real time PCR

All samples were carried out on a rotor gene 6000 Corbett detection system and quantified qPCR using SYBR®Premix Ex Taq™ II kit (TaKaRa, Japan) according to the manufacturer's instructions. Thermal cycling conditions were an initial activation step for 5 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. No template control (NTC) consisting of H_2O was included in each run. To verify specificity of PCR products, melting curve analysis was performed. Besides, PCR products were electrophoresed on 2% agarose gel to verify product sizes and specificity. For qPCR analysis, all samples were normalized to GAPDH. Forward and reverse primers sequences are as follows, respectively: *ARA*-F: 5′-TGCTGCACTTGAGCATTAGG-3′ and *ARA* -R: 5′-GCCTCCATGAAAAAGGATCA-3′ and GAPDH-F: 5′-GAAGGTGAAGGTCGGAGTC-3′ and GAPDH-R: 5′-GAAGATGGTGATGGGATTC-3′. The mean value in each triplicate was used to calculate relative lncRNA concentration (ΔCt = Ct mean lncRNA-Ct mean GAPDH). Expression fold changes were calculated using $2^{-\Delta\Delta Ct}$ methods [15]. The qPCR assays were performed in triplicate and the data were presented as the mean ± standard error of the mean (SEM).

Statistical analysis

The statistical analysis was performed by the GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA). Student's *t* test was conducted to compare *ARA* lncRNA expression in clinical samples. The Chi square test or Fisher's exact test was appropriately used for testing the relationship between categorical variables of breast and liver cancer. The level of statistical significance was set at $P < 0.05$.

Results

Expression of *ARA* lncRNA in patients' samples

Expression of *ARA* lncRNA was detectable in breast cancer and liver patients compared with healthy subjects. The expression of *ARA* lncRNA was measured in four groups: 15 healthy subject individuals from the breast cancer and liver community, 15 breast cancer patients and 15 liver patients by quantitate in patients with breast cancer and liver compared with healthy subjects. A significant over-expression of *ARA* lncRNA had been observed in both of breast and liver cancer compared with healthy groups ($P < 0.001$; Fig. 1). As present in Fig. 1, also there is a significant difference between the expression of *ARA* lncRNA in breast cancer versus expression of *ARA* lncRNA in liver cancer patients (P value < 0.05).

Correlations between *ARA* lncRNA expression and clinical characteristics

Correlations between clinical characteristics and *ARA* lncRNA expression evaluated by using the Chi square test and Fisher's exact test. Based on the median value of the *ARA* lncRNA expression in breast and liver cancer tissues, the patients were divided into two groups: cases with low *ARA* lncRNA expression and cases with high *ARA* lncRNA expression. High expression levels were classified as those that were above the median, while low expression levels were below the median. Table 1 indicated the relationships between *ARA* lncRNA expression and the clinic pathological characteristics of Iranian patients with BC.

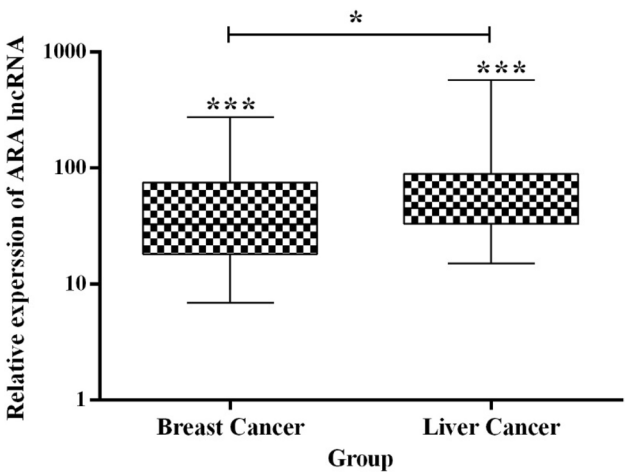

Fig. 1 Relative expression of *ARA* lncRNA in breast and liver cancer cases. Expression levels of the gene was evaluated by qPCR and compared to healthy subjects by ΔΔCt method. The numbers on Y-axis show fold changes and the star on the bars indicate significant change

Table 1 Correlation between *ARA* lncRNA expression and clinic pathological variables of breast cancer cases

Variables	Cases (%)	ARA lncRNA		*P* value
		Low	High	
Age 46.80 ± 2.57 (32–65)				0.447
≤47	53.3	33.3	20	
>47	46.7	20	26.7	
Tumor grade				0.310
I	6.7	6.7	0	
II	66.7	40	26.7	
III	26.7	6.7	20	
Nuclear grade				0.656
Low	7.1	7.1	0	
High and intermediate	28.6	14.3	14.3	
High	64.3	35.7	28.6	
Tumor stage				0.517
T1	40	13.3	26.7	
T2	13.3	6.7	6.7	
T3	40	26.7	13.3	
T4	6.7	6.7	0	
Tumor size (cm)				0.185
<2	73.3	46.7	26.7	
≥2	26.7	6.7	20	
Area of invasive component 4.09 ± 0.13 (0.7–9.5 cm^2)				0.714
<4	66.7	33.3	33.3	
≥4	33.3	20	13.3	
Tumor side				0.447
Right	53.3	33.3	20	
Left	46.7	20	26.7	
Margin				0.876
Free	73.3	40	33.3	
Involved	26.7	13.3	13.3	
Prevascular invasion				0.170
Negative	26.7	6.7	20	
Positive	73.3	46.7	26.7	
Preneural invasion				0.044
Negative	20	0	20	
Positive	80	53.3	26.7	

The Chi square test and Fisher's exact test showed that there was a significant correlation between *ARA* lncRNA expression and preneural invasion ($P = 0.044$). There were no significant associations between *ARA* lncRNA expression and other patients' clinic pathological characteristics ($P > 0.05$). Our results indicated that there were no significant correlation between *ARA* lncRNA expression and patients' clinic pathological characteristics in liver cancer patients ($P > 0.05$) (Table 2).

Table 2 Correlation between *ARA* lncRNA expression and clinic pathological variables of liver cancer

Variables	Cases (%)	ARA lncRNA		*P* value
		Low	High	
Age 55 ± 8.54 (47–74)				0.398
≤ 55	60	26.7	33.3	
> 55	40	26.7	13.3	
Sex				0.876
Male	73.3	40	33.3	
Female	26.7	13.3	13.3	
Tumor size (cm)				0.782
< 3	46.7	26.7	20	
≥ 3	53.3	26.7	26.7	
Angioinvasion				0.714
Yes	33.3	20	13.3	
No	66.7	33.3	33.3	
Differentiation				0.535
Poor differentiated	33.3	13.3	20	
Moderate differentiated	60	33.3	26.7	
Well differentiated	6.7	6.7	0	

Breast cancer-specific tumor marker

Receiver operating characteristic (ROC) curves were created and the area under the curve (AUC) was calculated to determine the ability of *ARA* lncRNA to differentiate between cancer cases and controls, by computation sensitivity and specificity for possible cutoff point of *ARA* lncRNA. ROC analysis determined the optimal cutoff value for *ARA* lncRNA to differentiate Breast cancer cases from controls. The sensitivity of circulating *ARA* lncRNA were specified to be 100% at the specificity of 100% with an area under the ROC curve of 1 (Fig. 2). In Fig. 2 also we indicated ROC curve for *ARA* lncRNA to differentiate liver cases from controls. We found the sensitivity of circulating *ARA* lncRNA: 100% at the specificity of 100% with an area under the ROC curve of 1.

Correlation between *ARA* lncRNA expression and patient survival

Association between the expression levels of *ARA* lncRNA with survival investigated through Kaplan–Meier analysis in order to assess the prognostic value of *ARA* lncRNA as biomarker for breast and liver cancer. We used the log-rank test in breast and liver cancer patients. The Cox proportional hazards regression model was also utilized to evaluate the predictive value of *ARA* lncRNA levels in breast and liver cancer patients. Overall survival (OS) was defined as the time between the date of surgery and date of death or last follow-up. Clinicopathological factors and overall survival were then analyzed in the high and low *ARA* lncRNA expression groups, but no significant differences were observed between groups in both breast and liver cancer ($P > 0.05$, Tables 3, 4; Fig. 3).

Discussion

In last decade, various reports have identified different lncRNAs with important regulatory parts in cancer initiation and progression [16–18]. Non-coding RNAs play critical roles in some aspect of cell biology [19, 20]. These molecules, such as mRNAs, produced by RNA polymerase II enzyme, capping process and polyadenylation [21]. In general, lncRNAs is contain about 80% of non-coding RNAs [22]. LncRNAs can act as oncogenes and tumor suppressor genes, so has shown research, which these molecules play an important role in oncogenes, angiogenesis, proliferation, migration, apoptosis and differentiation, or lncRNAs, greatly affect the malignant behavior of cancer [23].

Few systematic studies have focused on global regulation of lncRNAs in adriamycin-resistant cells [13]. Adriamycin known as a drug for chemotherapy that acts as a target for topoisomerase II poison. It is used to treat numerous types of cancer e.g. breast and liver cancer [24]. However, adriamycin resistance, making the cancer harder to treat. Jiang et al. [13] recently recognized several lncRNAs including

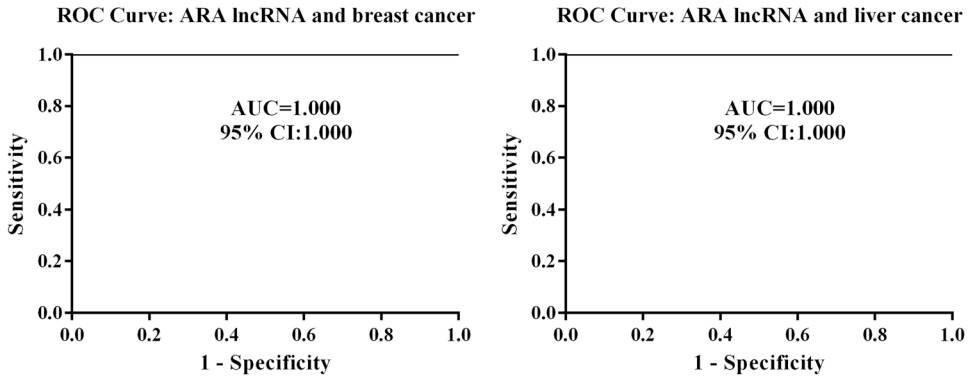

Fig. 2 Receiver-operating characteristic (ROC) curve analyses of *ARA* lncRNA signature to discriminate breast and liver cancer patients from normal controls

Table 3 Log rank test for all patients undergoing breast cancer

Variables	Overall survival		
	HR	95% CI	P
ARA (low vs. high)	0.520	0.1059–2.601	0.354
Age (≥47 vs. ≥47)	1.749	0.3501–8.657	0.506
Tumor grade (I–III vs. III)	0.560	0.07296–3.501	0.493
Nuclear grade (low vs. high and intermediate-high)	1.977	0.1596–40.67	0.517
Tumor stage (T1–T2 vs. T3–T4)	0.5745	0.1041–2.891	0.800
Tumor size (<2 cm vs. ≥2 cm)	0.460	0.04656–2.841	0.347
Area of invasive component (<4 cm^2 vs. ≥4 cm^2)	0.576	0.07625–3.827	0.378
Tumor side (right vs. left)	0.585	0.1185–2.937	0.525
Margin (free vs. involved)	0.507	0.05886–3.166	0.416
Prevascular invasion (negative vs. positive)	1.605	0.3264–8.170	0.566
Preneural invasion (negative vs. positive)	0.336	0.01890–1.809	0.166

Table 4 Log rank test for all patients undergoing liver cancer

Variables	Overall survival		
	HR	95% CI	P
ARA (low vs. high)	1.702	0.3887–7.950	0.361
Age (≥55 vs. ≥55)	0.321	0.05506–1.105	0.106
Sex (male vs. female)	0.366	0.04357–1.604	0.159
Tumor size (<3 cm vs. ≥3 cm)	0.370	0.08635–2.124	0.319
Angioinvasion (yes vs. no)	1.003	0.1945–5.180	0.997
Differentiation (poor vs. moderate and well)	0.505	0.1005–1.985	0.347

ARA lncRNA that showed various expression in adriamycin resistant breast and liver cancer cell lines. Over expression of *ARA* lncRNA has been found in adriamycin resistant MCF7 cells compared to the parental cell line. Breast cancer cell lines treatment with adriamycin led to an increase in *ARA* lncRNA expression. *ARA* lncRNA might be involved in self-sufficiency in estrogen signaling. Adriamycin resistant breast cancer cell lines restored inhibiting cellular proliferation and inducing apoptosis. *ARA* lncRNA may contribute a proliferative and survival advantage to adriamycin resistant cells [13].

Considering the reports of lncRNAs and cancer, in present study we aimed to explore *ARA* lncRNA expression in both breast and liver tumors. *ARA* lncRNA was reported to be upregulated in breast and liver cell lines [13]. The findings of our study revealed this upregulation in both breast and liver tissue samples. To the best of our knowledge, this is the first report to determine the expression of *ARA* lncRNA in breast and liver cancer in Iran. Nevertheless, these findings are preliminary and need to be validated in a larger population. Therefore, we could better analyze the suitability of ANCR as a potential diagnostic and prognostic biomarker with employing more samples, subdivided in different grades and stages of malignancies.

In Conclusion, we demonstrate the expression pattern of *ARA* lncRNA in both breast and liver tumors. LncRNAs and profiles based on lncRNAs may also become helpful in cancer gene therapy. Our data is the first report on different expression of *ARA* lncRNA in breast and liver cancers in IRAN, demonstrating a possible link between the expression level of *ARA* lncRNA and cell proliferation. Moreover, with further validation, *ARA* lncRNA can potentially be considered as a novel tumor biomarker with potential diagnostic, prognostic and therapeutic value. However, to

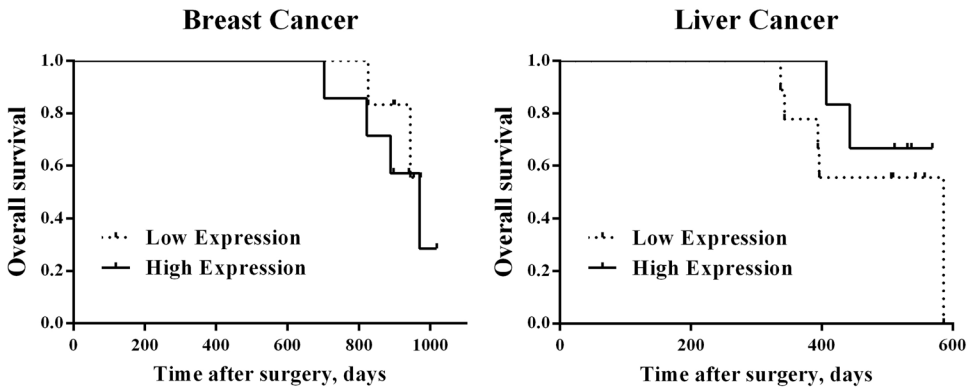

Fig. 3 Kaplan–Meier survival curves for associations of ARA with survival. ARA expression and overall survival (OS) in breast and liver cancer patients: ARA low versus high, P > 0.05 (log-rank test)

validate our findings, studies on various ethnic groups in a larger part of population may warranted the results.

Acknowledgements The authors are grateful to the staffs of research deputy of Shahrekord University of Medial Sciences and Cellular and Molecular Research Center, Shahrekord University of Medical Sciences.

Author contributions FR, MA, EM and SI; literature review, manuscript writing and data collection. AA; literature review and manuscript revision, EN, AA; manuscript revision and RA-N, MK; literature review.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care of human were followed.

References

1. Ilic M, Ilic I (2016) Epidemiology of pancreatic cancer. World J Gastroenterol 22:9694–9705. https://doi.org/10.3748/wjg.v22.i44.9694
2. Sighoko D, Hunt BR, Irizarry B et al (2018) Disparity in breast cancer mortality by age and geography in 10 racially diverse US cities. Cancer Epidemiol 53:178–183. https://doi.org/10.1016/j.canep.2018.02.003
3. Seyfried TN, Huysentruyt LC (2013) On the origin of cancer metastasis. Crit Rev Oncog 18:43–73
4. Chen W, Hoffmann AD, Liu H, Liu X (2018) Organotropism: new insights into molecular mechanisms of breast cancer metastasis. NPJ Precis Oncol 2:4. https://doi.org/10.1038/s41698-018-0047-0
5. Byler S, Goldgar S, Heerboth S et al (2014) Genetic and epigenetic aspects of breast cancer progression and therapy. Anticancer Res 34:1071–1077
6. Samulin Erdem J, Notø H, Skare Ø et al (2017) Mechanisms of breast cancer risk in shift workers: association of telomere shortening with the duration and intensity of night work. Cancer Med 6:1988–1997. https://doi.org/10.1002/cam4.1135
7. Kar P (2014) Risk factors for hepatocellular carcinoma in India. J Clin Exp Hepatol 4:S34–S42. https://doi.org/10.1016/j.jceh.2014.02.155
8. Pan H, Fu X, Huang W (2011) Molecular mechanism of liver cancer. Anticancer Agents Med Chem 11:493–499
9. Jiang K, Al-Diffalha S, Centeno BA (2018) Primary liver cancers-part 1: histopathology, differential diagnoses, and risk stratification. Cancer Control. https://doi.org/10.1177/1073274817744625
10. Kaikkonen MU, Lam MTY, Glass CK (2011) Non-coding RNAs as regulators of gene expression and epigenetics. Cardiovasc Res 90:430–440. https://doi.org/10.1093/cvr/cvr097
11. Lin C-P, He L (2017) Noncoding RNAs in cancer development. Annu Rev Cancer Biol 1:163–184. https://doi.org/10.1146/annurev-cancerbio-050216-034443
12. Schmitt AM, Chang HY (2016) Long noncoding RNAs in cancer pathways. Cancer Cell 29:452–463. https://doi.org/10.1016/j.ccell.2016.03.010
13. Jiang M, Huang O, Xie Z et al (2014) A novel long non-coding RNA-*ARA*: adriamycin resistance associated. Biochem Pharmacol 87:254–283. https://doi.org/10.1016/j.bcp.2013.10.020
14. Malhotra A, Jain M, Prakash H et al (2017) The regulatory roles of long non-coding RNAs in the development of chemoresistance in breast cancer. Oncotarget 8:110671–110684. https://doi.org/10.18632/oncotarget.22577
15. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta C_T$ method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262
16. Shahryari A, Rafiee MR, Fouani Y et al (2014) Two novel splice variants of ARA, ARA-S1, and ARA-S2 are coupregulated with SOX2 and OCT4 in esophageal squamous cell carcinoma. Stem Cells 32:126–134. https://doi.org/10.1002/stem.1542
17. Tian Y, Zhang X, Hao Y et al (2014) Potential roles of abnormally expressed long noncoding RNA UCA1 and Malat-1 in metastasis of melanoma. Melanoma Res 24:335–341. https://doi.org/10.1097/CMR.0000000000000080
18. He Y, Meng X-M, Huang C et al (2014) Long noncoding RNAs: novel insights into hepatocelluar carcinoma. Cancer Lett 344:20–27. https://doi.org/10.1016/j.canlet.2013.10.021
19. Zhou H-L, Luo G, Wise JA, Lou H (2014) Regulation of alternative splicing by local histone modifications: potential roles for RNA-guided mechanisms. Nucleic Acids Res 42:701–713. https://doi.org/10.1093/nar/gkt875
20. Soreq L, Guffanti A, Salomonis N et al (2014) Long non-coding RNA and alternative splicing modulations in Parkinson's leukocytes identified by RNA sequencing. PLoS Comput Biol 10:e1003517. https://doi.org/10.1371/journal.pcbi.1003517
21. Kung JTY, Colognori D, Lee JT (2013) Long noncoding RNAs: past, present, and future. Genetics 193:651–669. https://doi.org/10.1534/genetics.112.146704
22. Wapinski O, Chang HY (2011) Long noncoding RNAs and human disease. Trends Cell Biol 21:354–361
23. Kondo Y, Shinjo K, Katsushima K (2017) Long non-coding RNAs as an epigenetic regulator in human cancers. Cancer Sci 108:1927–1933
24. Cortés-Funes H, Coronado C (2007) Role of anthracyclines in the era of targeted therapy. Cardiovasc Toxicol 7:56–60. https://doi.org/10.1007/s12012-007-0015-3